AD_____

Award Number: DAMD17-02-1-0606

TITLE: Folate and Breast Cancer: Role of Intake, Blood Levels, and Metabolic Gene Polymorphisms

PRINCIPAL INVESTIGATOR: Martha J. Shrubsole, Ph.D.

CONTRACTING ORGANIZATION: Vanderbilt University Medical Center
Nashville, Tennessee 37232-1203

REPORT DATE: June 2004

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	June 2004	Annual Summary (1 Jun 2003 - 31 May 2004)

4. TITLE AND SUBTITLE
Folate and Breast Cancer: Role of Intake, Blood Levels, and Metabolic Gene Polymorphisms

5. FUNDING NUMBERS
DAMD17-02-1-0606

6. AUTHOR(S)
Martha J. Shrubsole, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Vanderbilt University Medical Center
Nashville, Tennessee 37232-1203

E-Mail: martha.shrubsole@mcmail.vanderbilt.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

The purpose is training in nutritional and molecular epidemiology to establish an independent investigator. The major hypothesis is that high folate intake is associated with a decreased breast cancer risk particularly among those with *MTHFR, MTR*, and *MTRR* polymorphisms. The specific aims are 1) methodological training in the analysis of gene-gene and gene-environment interactions by studying folate intake and metabolic gene polymorphisms in a population-based breast cancer case-control study, 2) training in cohort study methodology through designing and implementing a newly proposed nested case-control study of breast cancer to examine dietary and plasma folate, and metabolic gene polymorphisms, 3) nutrition and cancer biology coursework 4) field work of a breast cancer case-control study and 5) development of a grant proposal examining folate, global DNA methylation and uracil misincorporation in breast cancer risk. To date, the major results are the *MTHFR 677TT* genotype and low folate intake is associated with an increased risk of breast compared to high intake and the *677CC* genotype. We also found *677CC* was associated with poorer survival from breast cancer among women with late-stage disease who had survived at least one year post-diagnosis. The investigator has also completed coursework, training in methodology, and field work experience.

14. SUBJECT TERMS
Epidemiology, nutrition, gene-environment interaction, prevention gene-gene interaction, DNA methylation

15. NUMBER OF PAGES
34

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Folate and Breast Cancer: Role of Intake, Blood Levels, and Metabolic Gene Polymorphisms.

INTRODUCTION

Folate, a B vitamin found naturally in many food sources particularly in dark green leafy vegetables, is essential for regenerating methionine, the methyl donor for DNA methylation, and for producing the purines and pyrimidine thymidylate required for DNA synthesis and repair. Evidence for its potential role in carcinogenesis is encouraging. Several genes involved in the metabolism of folate have known polymorphisms and the combined effect of these polymorphisms with folate intake may affect breast cancer risk. MTHFR irreversibly converts 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which provides the methyl group for the de novo methionine synthesis and DNA methylation. Two common polymorphisms in the *MTHFR* gene have been identified both of which result in decreased MTHFR activity (C677T, A1298C). Methionine synthase, a vitamin B12-dependent enzyme that converts homocysteine to methionine by the transfer of a methyl group from 5-methyltetrahydrofolate, is encoded by the *MTR* gene and a polymorphism has been identified (A2756G). Methionine synthase reductase reductively activates methionine synthase from its inert to reactive form. The gene, *MTRR*, has been identified along with a polymorphism (A66G). *MTHFR* and *MTR* polymorphisms have been associated with reduced colorectal cancer risk. A small hospital-based case-control study found an increased risk with *MTHFR* 677T and no association with *MTR*, results not consistent with the more extensive colorectal cancer results. No breast cancer study has evaluated the role of *MTRR*..**Purpose:** The specific aims of this postdoctoral training proposal are 1) training in the analysis of gene-gene and gene-environment interactions by studying folate intake and folate metabolic gene polymorphisms (MTHFR, MTR, MTRR) in a population-based breast cancer case-control study (approximately 3000 subjects), 2) training in the methodology of cohort studies through a newly proposed nested case-control study of breast cancer (350 pairs) examining folate intake, plasma folate, and metabolic gene polymorphisms, 3) coursework in nutrition and cancer biology and 4) development of a grant proposal examining folate and global DNA methylation in breast cancer risk. **Scope:** Most established risk factors for breast cancer are very difficult to modify, therefore, identifying modifiable factors is essential to prevent the disease globally. The US and Canada currently fortify all cereal grain foods with folic acid, although most other countries do no. A serendipitous result may be an eventual decrease in breast cancers. It is necessary, therefore, to assess the relationship between folate and breast cancer so that high-risk groups may be targeted and international breast cancer incidence decrease.

BODY

Approved Statement of Work

Task 1. Undergo course training in nutrition and molecular biology, Months 1-22:

a. Take 1 course in the Vanderbilt Department of Molecular Biology (Fall Semester, 2002), Introduction to Cell Biology: Months 6-10.

b. Take 1 course in the Vanderbilt School of Medicine (Spring Semester 2003), Introduction to Clinical Nutrition: Months 11-12.

c. Take 1 course in the Vanderbilt Department of Molecular Biology (Spring Semester 2003), Cancer Biology: Months 11-15.

d. Take 1 course in the Vanderbilt Department of Biochemistry (Fall Semester, 2003), Molecular Aspects of Cancer Research: Months 18-22.

1a. Introduction to Cell Biology was completed in Fall Semester 2003.

1b. Introduction to Clinical Nutrition will be replaced with an independent study of nutrition.

1c. Cancer Biology was completed in December 2001.

1d. Molecular Aspects of Cancer Research was replaced with the American Association for Cancer Research Pathobiology of Cancer Edward A. Smuckler Memorial Workshop in July 13-20 2003. I was one of 100 people chosen as a participant.

In addition to the specific courses listed above, the American College of Epidemiology Molecular Genetics for Epidemiologists: from the Basics to Advanced Topics Workshop was completed in June 2003.

Folate and Breast Cancer: Role of Intake, Blood Levels, and Metabolic Gene Polymorphisms.

Task 2. Undergo training in the analysis of gene-gene and gene-environment interactions and the associations of folate intake and folate metabolizing gene polymorphisms using data from a population-based case-control study of 3000 subjects in Shanghai: Months 1-24

a. Analyze the association between MTHFR polymorphisms and breast cancer risk and prepare a manuscript to report the findings: Months 1-10.

b. Analyze and publish the joint effect of MTHFR, MTR, and MTRR polymorphisms, folate intake, and breast cancer risk: Months 10-18.

2a. The manuscript was published in *Cancer Epidemiology Biomarkers & Prevention* in February 2004. A copy of this manuscript is included in Appendix 1. We found that *MTHFR* genotypes were not associated with breast cancer risk, however, *MTHFR C677T* genotypes appeared to modify the association between folate and breast cancer risk. I also evaluated the MTHFR genotypes in relation to survival from breast cancer in this study. We found that the *MTHFR C677T* genotype was associated with an increased risk of death for women who were initially diagnosed with a late-stage cancer and who had survived at least one year after initial diagnosis. This manuscript was submitted to *Clinical Cancer Research* in April 2003 and is in review.

2b. The *MTRR* assays have recently been completed. The *MTR* data will become available in July 2004. At that time, the manuscript will be prepared.

Task 3. Undergo training in the methodology of cohort studies and to evaluate the association of folate with breast cancer risk using data from a prospective cohort study of 75, 000 Chinese women in Shanghai: Months 1-36.

a. Design a nested case-control study (350 matched pairs) within the Shanghai Women's Health Study for the prospective evaluation of folate intake, plasma folate, and metabolic gene polymorphisms in relation to breast cancer risk: Months 1-19.

b. Prepare blood samples for relevant assays: Months 1-19.

c. Analyze and publish the relationship between folate intake (all 75, 000 women), plasma folate (700 subjects in a nested case-control study) and breast cancer risk. Months 21-26.

d. Analyze and publish the relationship between metabolic gene polymorphisms and breast cancer risk: Months 26-36.

e. Analyze the joint effect of metabolic gene polymorphisms, plasma and dietary folate, and breast cancer risk: Months 26-36.

3a. Follow-up of all participants is on-going. At this time, 248 breast cancer cases have been identified, of which, 184 have a blood sample for folate analysis. The nested-case control study has been designed for these 184 cases. Other cases and controls will be accrued prospectively.

3b. Blood samples have been prepared and DNA isolated for the folate and gene polymorphism assays. The assays will begin in the late summer 2004.

3c-e. Data for the analyses will be available in late 2004. At that time, the stated analyses will be done.

Task 4. Undergo training in implementation and administration of breast cancer epidemiological studies by participating in the field work of the Nashville Breast Health Study, a new case-control study. Months 1-36.

a. Assist in the development of study instruments, materials, and procedures: Months 1-6.

b. Participate in subject identification and recruitment: Months 3-36.

c. Prepare manuscripts for publication: Months 26-36.

4a. In 2002-2003, I developed and modified several study instruments including telephone questionnaire, call logs, and other procedural forms. I have developed protocols for patient recruitment, random digit dialing, interviewer training and other interviewer procedures. I have designed a database for patient tracking and data entry and several reports to monitor study progress.

4b. I continue to be involved in project management of the Nashville Breast Health Study.

Folate and Breast Cancer: Role of Intake, Blood Levels, and Metabolic Gene Polymorphisms.
Task 5: Prepare a grant proposal for continuation. Months 28-34.

a. Develop and submit a grant proposal to expand the sample size of the nested case-control study to evaluate folate, global DNA methylation, and uracil misincorporation in lymphocytes in relation to breast cancer risk.

5. I have recently begun accumulating literature on DNA methylation and speaking with basic scientists in preparation for Task 5.

KEY RESEARCH ACCOMPLISHMENTS

- **July 2003:** Attended AACR Pathobiology of Cancer Workshop
- **Fall 2003:** Attended Cell Biology in the Vanderbilt Department of Cell Biology
- **October 2003:** The nested breast cancer case-control study of the Shanghai Women's Health Study was designed
- **February 2004:** Published the results from the *MTHFR* genotype and breast cancer risk. *MTHFR* genotypes were not associated with breast cancer risk, however, *MTHFR C677T* genotypes appeared to modify the association between folate and breast cancer risk (Appendix 1).
- **Winter 2003/04:** *MTHFR* genotype and survival analyses completed for the Shanghai Breast Cancer Study. *MTHFR 677TT* genotype was associated with poor survival among women who had received chemotherapy and had late-stage disease (Appendix 2).
- **Spring 2004:** *MTRR* genotyping completed for the Shanghai Breast Cancer Study; DNA samples prepared for *MTR* genotyping; blood samples for folate analysis for the breast cancer nested case-control study in the Shanghai Women's Health Study
- **On-going:** project management of the Nashville Breast Health Study; 714 participants have been recruited

REPORTABLE OUTCOMES

1. **Shrubsole, MJ**, Jin F, Dai Q, Shu XO, , Hebert JR, Niu Q, Gao YT, Zheng W .MTHFR polymorphisms, dietary folate intake, and breast cancer risk: Results from the Shanghai Breast Cancer Study. 2004. *Cancer Epidemiology, Biomarkers, and Prevention.* 13(2):190-6. *MTHFR* genotypes were not associated with breast cancer risk, however, *MTHFR C677T* genotypes appeared to modify the association between folate and breast cancer risk (Appendix 1).
2. **Shrubsole MJ,** Shu XO, Ruan ZX, Cai Q, Cai H, Gao YT, Zheng W. *MTHFR* genotypes and breast cancer survival: A report from the Shanghai Breast Cancer Study. Presented as a poster at the SNPs, Haplotypes, and Cancer: Applications in Molecular Epidemiology of the American Association for Cancer Research. 2003.
3. **Shrubsole MJ,** Shu XO, Ruan ZX, Cai Q, Cai H, Niu Q, Gao YT, Zheng W. *MTHFR* genotypes and breast cancer survival after surgery and chemotherapy: A report from the Shanghai Breast Cancer Study. 2004. *Clinical Cancer Research* (submitted). (Appendix 2)
4. Attendee: American Association for Cancer Research Pathobiology of Cancer Workshop.

CONCLUSIONS

Results from the *MTHFR* and breast cancer risk manuscript indicate that *MTHFR* genotype alone is not associated with breast cancer risk. However, *MTHFR* genotype may affect the degree to which folate is protective in breast cancer risk. This is one of the first and largest studies to examine this association. It will be important to verify this relationship in the nested case-control study supported by this grant. This study has made good progress and the laboratory assays will begin soon. The analysis of *MTHFR* genotype and breast cancer survival indicates that *MTHFR* genotype is a risk factor for death from breast cancer among women who have had chemotherapy and have late-stage disease. Future studies will be necessary to evaluate whether the type of chemotherapy received affects this association.

I have become involved in the study design and management of two additional cancer epidemiology studies as a result of my experience with the Nashville Breast Health Study. This will further expand my ability to become an independent investigator and these new studies will be used to strengthen the grant application in Task 5.

REFERENCES

1. **Shrubsole, MJ**, Jin F, Dai Q, Shu XO, , Hebert JR, Niu Q, Gao YT, Zheng W .MTHFR polymorphisms, dietary folate intake, and breast cancer risk: Results from the Shanghai Breast Cancer Study. 2004. *Cancer Epidemiology, Biomarkers, and Prevention.* 13(2):190-6.(Appendix 1)
2. **Shrubsole MJ,** Shu XO, Ruan ZX, Cai Q, Cai H, Niu Q, Gao YT, Zheng W. *MTHFR* genotypes and breast cancer survival after surgery and chemotherapy: A report from the Shanghai Breast Cancer Study. 2004. *Clinical Cancer Research* (submitted). (Appendix 2)

Appendix 1

Shrubsole, MJ, Jin F, Dai Q, Shu XO, , Hebert JR, Niu Q, Gao YT, Zheng W .
MTHFR polymorphisms, dietary folate intake, and breast cancer risk: Results from the Shanghai Breast Cancer Study. 2004. *Cancer Epidemiology, Biomarkers, and Prevention.* 13(2):190-6.

MTHFR Polymorphisms, Dietary Folate Intake, and Breast Cancer Risk: Results from the Shanghai Breast Cancer Study

Martha J. Shrubsole,[1] Yu-Tang Gao,[2] Qiuyin Cai,[1] Xiao Ou Shu,[1] Qi Dai,[1] James R. Hébert,[3] Fan Jin,[2] and Wei Zheng[1]

[1]Department of Medicine and Vanderbilt-Ingram Cancer Center, Vanderbilt University, Nashville, Tennessee; [2]Department of Epidemiology, Shanghai Cancer Institute, Shanghai, People's Republic of China; and [3]Department of Epidemiology and Biostatistics, Norman J. Arnold School of Public Health, University of South Carolina, Columbia, South Carolina

Abstract

Folate plays an important role in DNA methylation, synthesis, and repair; intake has been associated with breast cancer. The folate-metabolizing enzyme, methylenetetrahydrofolate reductase (MTHFR) is polymorphic at nucleotides 677 (C→T) and 1298 (A→C), resulting in allozymes with decreased activity. We evaluated these two common polymorphisms and their effects on the folate intake and breast cancer risk association in a population-based case-control study of 1144 breast cancer cases and 1236 controls using a PCR-RFLP-based assay. All subjects completed in-person interviews, which included a food frequency questionnaire. Unconditional logistic regression models were used to calculate odds ratios and their 95% confidence intervals, after adjusting for potential confounding factors. Cases and controls were similar in the distribution of *MTHFR* polymorphisms at codons 677 (41.4% cases and 41.8% controls carried the *T* allele) and 1298 (17.6% cases and 17.5% controls carried the *C* allele). An inverse association of breast cancer risk with folate intake was observed in all genotype groups, particularly among subjects with the *677TT* genotype. Compared with those with the *677CC* genotype and high folate, the adjusted odds ratios (95% confidence intervals) associated with low folate intake were 1.94 (1.15–3.26), 2.17 (1.34–3.51), and 2.51 (1.37–4.60) for subjects who had *CC*, *CT*, and *TT* genotypes (p for interaction, 0.05). No modifying effect of A1298C genotypes on the association of folate intake with breast cancer risk was observed. Results of this study suggest that the *MTHFR C677T* polymorphisms may modify the association between dietary folate intake and breast cancer risk.

Introduction

Folate is involved in DNA methylation, synthesis, and repair. Low intake of folate may increase risk for several cancers, including breast cancer (1, 2). The enzyme methylenetetrahydrofolate reductase (MTHFR) irreversibly catalyzes 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, the donor for the remethylation of homocysteine to methionine, the precursor for the universal methyl donor, *S*-adenosylmethionine (3, 4). Folate that is not converted through this pathway can be used for purine synthesis or the conversion of uracil to thymine, which is used for DNA synthesis and repair (5).

Two common polymorphisms in the *MTHFR* gene have been characterized (6, 7). The 677C → T polymorphism codes for an alanine to valine substitution in the N-terminal catalytic domain and results in an allozyme with ~65% and ~30% of the wild-type homozygote activity for heterozygotes and homozygotes of the variant allele, respectively (6, 8). The A→ C polymorphism at nucleotide 1298 codes for an alanine to glutamine substitution in the C-terminal regulatory domain (7). Individuals homozygous for the *1298C* allele have approximately the same enzyme activity as those heterozygous for the *677T* allele (7, 8).

The C677T polymorphism has been examined in relation to several cancers (2, 9). In most studies of colorectal neoplasms, the *MTHFR 677TT* genotype has been associated with an overall reduction in risk, reduced risk among those with higher intakes of folate (10–13), or increased risk among those with lower folate intakes (13–15). *MTHFR* has not been as well studied in relation to breast cancer risk. Only three small studies have evaluated the association between *MTHFR* genotype and breast cancer (16–18). The results from these studies have been inconsistent. Only one study assessed both the *C677T* and *A1298C* polymorphisms and their possible joint effect with folate intake (18). However, in that study, only 60 cases were included. We reported recently that folate intake was inversely associated with breast cancer risk in a large population-based, case-control study among Chinese women in Shanghai (19). In an extension of these results, we investigated whether this association may be modified by *MTHFR* genotypes.

Materials and Methods

The Shanghai Breast Cancer Study is a population-based, case-control study conducted in urban Shanghai, China during 1996–1998. This study was approved by the committees for the use of human subjects in all collaborating institutions. Detailed study methods have been published previously (20).

Subjects. All incident breast cancer cases newly diagnosed during the study period and meeting the eligibility criteria were identified through a rapid case-ascertainment system supple-

Received 8/8/03; revised 9/15/03; accepted 9/26/03.

Grant support: This research was supported by United States Public Health Service Grant RO1CA64277 from the National Cancer Institute. Dr. Shrubsole is supported, in part, by DAMD17-02-1-0606 from the United States Army Medical Research and Materiel Command.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Wei Zheng, Center for Health Services Research, Vanderbilt University Medical Center, Nashville, TN 37232-8300. Phone: (615) 936-0682; Fax: (615) 936-1269 E-mail: wei.zheng@vanderbilt.edu.

mented by the Shanghai Cancer Registry and were approached for participation in the study. Eligibility criteria for the study were as follows: 25–64 years of age, resident of urban Shanghai, no previous history of any cancer, and alive at the time of interview. In all, 1602 eligible cases were identified, of whom 1459 (91.1%) completed in-person interviews. The median interval from cancer diagnosis to the in-person interview was 64 days. With the exception of a breast cancer diagnosis, controls had inclusion criteria identical to those of the cases and were frequency matched on age (5 years intervals) to the expected age distribution of the cases. In all, 1724 eligible controls were randomly selected from the Shanghai Resident Registry. Of these, 1556 (90.3%) completed in-person interviews.

Data and Biological Sample Collection. All subjects completed an in-person interview that used a structured questionnaire and incorporated anthropometric measurements. Dietary intakes were assessed using a 76-item food frequency questionnaire (FFQ). In a recent validation study of the FFQ among 200 women, we found that the FFQ captured >86% of food intake in Shanghai (21). Each subject was asked about the frequency that a specific food was eaten (daily, weekly, monthly, yearly, or never), followed by a question on the amount typically eaten. Dietary intakes of total folate and folate cofactors were derived from the FFQ by summing the product of the micronutrient content of each food item, usual portion eaten, and frequency of consumption. Because of the lack of folate data in the Chinese food composition database, an identical (82%) or equivalent (17%) item from the United States Department of Agriculture food composition database was used to determine micronutrient level (19). To assess the comparability of the Chinese and United States Department of Agriculture food composition databases, we evaluated the correlation of three other water-soluble vitamins (vitamin C, riboflavin, and niacin) and found excellent correlation; all Pearson correlation coefficients were $r \geq 0.91$ or higher, providing ancillary support for the validity of derived folate data in this study. In a validation study of the FFQ among 200 women, the FFQ was administered twice in a year and Pearson correlation for folate was $r = 0.36$. Blood samples were collected from 1193 (82%) cases and 1310 (84%) controls and used in this study for genotyping assays.

Laboratory Methods. Genomic DNA was extracted from blood samples with the Puregene DNA isolation Kit (Gentra Systems, Minneapolis, MN) following the protocol of the manufacturer. Genotyping for the *MTHFR C677T* and *A1298C* polymorphisms were performed using PCR-RFLP methods reported by Frosst *et al.* (6) and Weisberg *et al.* (7), with minor modifications. The primers for *C677T* analysis were 5′-TGAAGGAGAAGGTGTCTGCGGGA-3′ (exonic) and 5′-AGGACGGTGCGGTGAGAGTG-3′ (intronic). The primers for *A1298C* analysis were 5′-GGGAGGAGCTGACCAGTG-CAG-3′ and 5′-GGGGTCAGGCCAGGGGCAG-3′. The PCR reactions were performed in a Biometra TGradient Thermocycler. Each 20 μl of PCR mixture contained 10 ng DNA, 1× PCR buffer [50 mM KCl, 10 mM Tris-HCl (pH 9.0)], 1.5 mM $MgCl_2$, 0.2 mM each of deoxynucleoside triphosphate, 0.5 mM of each primer, and 1 unit of *Taq*DNA polymerase. The reaction mixture was initially denatured at 94°C for 3 min. For *C677T* polymorphisms, PCR was performed in 30 cycles of 94°C for 45 s, 65°C for 45 s, and 72°C for 45 s. For A1298C polymorphisms, PCR was performed in 35 cycles of 94°C for 45 s, 65°C for 45 s, and 72°C for 45 s. The PCR was completed by a final extension cycle at 72°C for 7 min.

For *C677T* polymorphisms, each PCR product (10 μl) was digested with 10 units of *Hinf* I at 37°C for 3 h. The DNA fragments were then separated using 3% agarose gel and detected by ethidium bromide staining. The C→T substitution at nucleotide 667 creates a *Hinf* I digestion site. The PCR product (198 bp) with the *T* allele was digested to two fragments (175 bp and 23 bp), whereas the PCR product with wild-type C allele cannot be cut by *Hinf* I. For *A1298C* polymorphisms, each PCR product (10 μl) was digested with 5 units of *Fnu4H* I at 37°C for 3 h, followed by 3% agarose gel electrophoresis and ethidium bromide staining. The A→C substitution at nucleotide 1298 creates a *Fnu4H* I site. The PCR product (138 bp) with *C* allele was digested to two fragments (119 bp and 19 bp), whereas the PCR product with wild-type A allele cannot be cut by *Fnu4H* I.

Quality-control samples were included in various batches of samples assayed for the polymorphisms. The consistency rate was 98.5% in 119 quality-control samples that were repeated in the genotyping assays with their identities unknown to laboratory staff. Excluding a few subjects for whom sufficient DNA was not available or for whom the genotyping assay failed, genotyping data were obtained from 1112 cases and 1160 controls for *C677T* and 1121 cases and 1208 controls for *A1298C* polymorphisms. Because few women in the study consumed alcohol, a factor that may increase folate requirements, and because the data on the folate content of vitamins were not available, all analyses involving folate or its cofactors were limited to the cases (92.0%) and controls (91.1%) who were known not to consume alcohol regularly and not to take vitamin supplements.

Data Analysis. Odds ratios (ORs) were used to measure the association of breast cancer risk with *MTHFR* genotype. Unconditional logistic regression models were used to obtain maximum likelihood estimates of the ORs and their 95% confidence intervals (CIs), after adjusting for potential confounding variables. Risk factors previously identified as having an independent association with breast cancer were controlled in all models. These included age, personal history of fibroadenoma, age at first live birth, physical activity, waist-to-hip ratio, and daily meat intake. Age was included as a continuous variable throughout, and categorical variables were treated as indicator variables in the model. Quartile and tertile distributions of dietary intakes among controls were used to categorize all dietary intake variables. In the analyses including dietary factors, energy adjustment was performed using the standard multivariate method (22). Tests for trend were performed by entering categorical variables as continuous. Stratified analyses were used to evaluate the potential modifying effect of age, menopausal status, and folate and folate cofactor intakes on breast cancer risk associated with *MTHFR* genotypes and of *MTHFR* genotypes on breast cancer risk associated with folate intake. Tests for multiplicative interaction were done by including multiplicative variables in the logistic model and performing the likelihood ratio test. All statistical tests were based on two-sided probabilities using SAS, Version 8.2 (SAS Institute, Inc., Cary, NC).

Results

Comparisons between cases and controls on select demographic factors, established risk factors, and dietary factors are presented in Table 1. Cases were, in general, more highly educated, more likely to have a history of fibroadenoma, younger at menarche, older at first live birth and menopause, less likely to be physically active, and more likely to have a higher body mass index and waist-to-hip ratio than controls.

Table 1 Comparison of cases and controls by selected descriptive characteristics, Shanghai Breast Cancer Study, 1996–1998

Subject characteristics	Cases (n = 1144)	Controls (n = 1236)	P^a
Age, yr (mean ± SD)	46.4 ± 9.9	46.7 ± 8.8	0.42
Education, %			
No formal education	3.8	6.0	
Elementary school	8.5	8.6	
Middle or high school	75.8	75.3	
College or above	12.0	10.1	<0.05
Breast cancer in first-degree relative, %	3.4	2.4	0.15
Ever had breast fibroadenoma, %	9.7	5.2	<0.01
Age at menarche (yr)	14.5 ± 1.6	14.7 ± 1.7	<0.01
Ever had a live birth, %	94.9	95.9	0.27
Number of live births, mean ± SD	1.5 ± 0.8	1.5 ± 0.9	0.19
Age at first live birth, yr (mean ± SD)	26.8 ± 4.1	26.2 ± 3.8	<0.01
Postmenopausal, %	33.3	36.3	0.13
Age at menopause, yr (mean ± SD)	48.2 ± 4.6	47.4 ± 5.0	0.03
Physically active past 10 yr, %	19.3	25.8	<0.01
Body mass index, kg/m^2 (mean ± SD)	23.6 ± 3.4	23.2 ± 3.4	0.02
Waist-to-hip ratio, mean ± SD	0.81 ± 0.06	0.80 ± 0.06	<0.01
Daily animal food intake, g (mean ± SD)	90.4 ± 61.8	79.4 ± 50.1	<0.01
Daily plant food intake, g (mean ± SD)	501 ± 275	496 ± 278	0.73
Daily folate intake, μg (mean ± SD)	287 ± 141	303 ± 179	0.02
Daily methionine intake, g (mean ± SD)	1.72 ± 0.60	1.65 ± 0.56	<0.01
Daily vitamin B_{12} intake, μg (mean ± SD)	4.77 ± 4.11	4.69 ± 4.20	0.66
Daily vitamin B_6 intake, mg (mean ± SD)	1.83 ± 0.60	1.77 ± 0.57	0.03
Daily energy intake, kcal (mean ± SD)	1875 ± 467	1852 ± 459	0.23

[a] For χ^2 test (categorical variables) or t test (continuous variables).

Cases also had higher average daily intakes of animal foods, methionine, and vitamin B_6 and lower average daily intake of folate than controls.

The frequencies of *MTHFR* alleles and genotypes by case-control status and the association between *MTHFR* genotypes and breast cancer risk are presented in Table 2. The frequencies of the *677T* and *1298C* alleles were 0.41 and 0.18, respectively, among the controls. These were virtually identical to the frequency among the cases. Among the controls, the distributions of the *MTHFR* genotypes did not differ from the predicted distribution under Hardy-Weinberg equilibrium (P = 0.44 for the *C677T* polymorphisms and P = 0.58 for the *A1298C* polymorphisms). Risk of breast cancer did not differ statistically for the *C677T* or *A1298C* genotypes or for their combination. Similar associations were observed in analyses stratified by age and menopausal status (data not shown in table).

The joint association of *MTHFR* genotype and dietary folate intake with breast cancer risk is presented in Table 3. Low intake of folate was associated with an increased risk of breast cancer among all genotypes, particularly subjects with the *TT* genotype (OR = 2.51; 95% CI: 1.37–4.60) There was a significant multiplicative interaction between folate intake and *C677T* polymorphism in relation to breast cancer risk (P = 0.05). Elevated ORs were observed to be associated with folate

Table 2 *MTHFR* genotype frequencies and adjusted odds ratios (ORs) for breast cancer among Chinese women, Shanghai Breast Cancer Study, 1996–1998

Genotype[a]	Cases, n (%)	Controls, n (%)	Age-adjusted OR (95% confidence interval)	Multi-adjusted OR (95% confidence interval)
C677T				
CC	374 (33.6)	387 (33.4)	1.00 (reference)	1.00 (reference)
CT	555 (49.9)	577 (49.7)	1.00 (0.83–1.20)	1.01 (0.84–1.22)
TT	183 (16.5)	196 (16.9)	0.97 (0.76–1.24)	0.97 (0.76–1.25)
A1298C				
AA	768 (68.5)	824 (68.2)	1.00 (reference)	1.00 (reference)
AC	311 (27.7)	344 (28.5)	0.97 (0.81–1.16)	0.96 (0.80–1.16)
CC	42 (3.8)	40 (3.3)	1.13 (0.72–1.75)	1.14 (0.73–1.79)
Combined				
A1298C-AA				
C677T-CC	196 (18.0)	180 (15.9)	1.00 (reference)	1.00 (reference)
C677T-CT	375 (34.4)	410 (36.2)	0.84 (0.66–1.07)	0.85 (0.66–1.09)
C677T-TT	179 (16.4)	184 (16.3)	0.89 (0.67–1.19)	0.89 (0.67–1.20)
A1298C-AC/CC				
C677T-CC	171 (15.7)	203 (17.9)	0.77 (0.58–1.03)	0.77 (0.57–1.02)
C677T-CT/TT	168 (15.4)	155 (13.7)	0.99 (0.74–1.34)	1.01 (0.75–1.36)

[a] The frequencies of the *677T* allele were 41.4% in cases and 41.8% in controls (P = 0.81) and the frequencies of the *1298C* allele were 17.6% in cases and 17.5% in controls (P = 0.95).

[b] All ORs are adjusted for age, waist-to-hip ratio, age at first live birth, physical activity, menopausal status, and total meat intake.

Table 3 Joint association of *MTHFR* genotype and folate intake with breast cancer risk among Chinese women, Shanghai Breast Cancer Study, 1996–1998

Genotype	Daily Folate Intake[a]								*P* for trend
	Q_4 (High)		Q_3		Q_2		Q_1		
	Cases/ controls	Adjusted OR (95% CI)[a]	Cases/ controls	Adjusted OR (95% CI)[a]	Cases/ controls	Adjusted OR (95% CI)[a]	Cases/ controls	Adjusted OR (95% CI)[a]	
C677T[b]									
CC	69/88	1.00 (reference)	96/86	1.76 (1.12–2.77)	90/91	1.75 (1.08–2.83)	81/86	1.94 (1.15–3.26)	0.02
CT	103/117	1.16 (0.76–1.77)	135/142	1.50 (0.99–2.29)	133/137	1.73 (1.11–2.70)	145/136	2.17 (1.34–3.51)	0.06
TT	29/53	0.70 (0.40–1.23)	47/44	1.66 (0.97–2.85)	49/39	2.17 (1.23–3.81)	47/38	2.51 (1.37–4.60)	0.003
P for trend		0.51		0.71		0.49		0.31	
A1298C[c]									
AA	140/192	1.00 (reference)	184/192	1.59 (1.15–2.20)	195/185	1.94 (1.36–2.76)	194/186	2.18 (1.46–3.25)	0.0006
AC/CC	63/81	1.05 (0.70–1.57)	92/84	1.80 (1.22–2.67)	79/91	1.59 (1.04–2.44)	85/92	1.94 (1.23–3.05)	0.18
A1298C-AA[d]									
C677T-CC	42/45	1.00 (reference)	43/43	1.26 (0.68–2.34)	48/38	1.81 (0.95–3.44)	43/40	1.85 (0.94–3.67)	0.07
C677T-CT	67/81	0.86 (0.49–1.48)	94/101	1.18 (0.69–2.01)	90/98	1.35 (0.77–2.36)	100/98	1.74 (0.96–3.16)	0.08
C677T-TT	29/52	0.56 (0.30–1.05)	45/41	1.40 (0.75–2.60)	48/36	1.87 (0.98–3.57)	46/36	2.16 (1.09–4.28)	0.002
P for trend		0.13		0.63		0.81		0.70	

[a] All ORs are adjusted for age, waist-to-hip ratio, age at first live birth, physical activity, and total energy, meat, vitamin B_{12}, vitamin B_6, and methionine intakes. OR, odds ratio; CI, confidence interval.
[b] *P* for interaction, 0.048.
[c] *P* for interaction, 0.71.
[d] *P* for interaction, 0.06.

intake regardless of *A1298C* genotype, although the trend was statistically significant in only the *AA* group. To examine further the *C677T* association, analyses were restricted to *1298AA* individuals because of a small sample size for the *AC* and CC genotypes. Again, low intake of folate was associated with increased risk for all genotypes, and the increased risk was greatest among those with *677TT* genotype (OR = 2.16, 95% CI: 1.09–4.28, p for trend = 0.002, p for interaction = 0.06). There was a suggestive reduced risk associated with the *TT* genotype among those with high folate intake, although the OR was not statistically significant.

The joint associations of *MTHFR C677T* genotypes and folate intake with breast cancer risk are presented in Table 4, after stratifying by folate cofactor intake. With the exception of the high vitamin B_6 stratum, low folate intake was associated with an elevated risk of breast cancer, and the association appeared stronger among subjects with the *TT* genotypes. None of the tests for multiplicative interactions, however, was statistically significant, perhaps because of a small sample size in these stratified analyses.

Discussion

We found in this case-control study that there was no statistically significant association between the risk of breast cancer and *MTHFR C677T* or *A1298C* genotypes. However, *MTHFR C677T* genotype was a statistically significant effect modifier of the association between folate intake and breast cancer risk. Among those with the *677TT* genotype, low folate intake was associated with a more substantial increased risk than those with other genotypes. These are novel findings consistent with the possible role of MTHFR and folate in the etiology of cancer.

MTHFR polymorphisms have not been adequately investigated in relation to breast cancer risk. Only three previous small studies have examined *MTHFR* polymorphisms and breast cancer risk (16–18). In the first, a study among Jewish women, *MTHFR C677T* genotype was determined in 491 women with sporadic (n = 355) or hereditary (n = 136) breast and/or ovarian cancer and in 69 asymptomatic BRCA1/2 mutation carriers (16). The prevalence of the *T* allele was not significantly different between sporadic cases and the asymptomatic carriers, women diagnosed at a young and older age, and *BRCA1/2* carriers with and without cancer. The prevalence of the *T* allele was more frequent among women with bilateral breast cancer or with both breast and ovarian cancers than among women with only unilateral breast cancer. In the second study, a hospital-based, case-control study among postmenopausal Caucasian women (149 cases and 171 controls), it was reported that the *MTHFR 677T* (*val*) allele was more prevalent in cases than controls (17), which is in contrast to the results from the third case-control study conducted in the United Kingdom (62 cases, 66 controls), the only previous study that reported risk of breast cancer associated with both the *C677T* and *A1298C* polymorphisms (18). The British study reported breast cancer risk was reduced among those homozygous for the *677T* allele (OR = 0.39; 95% CI: 0.12–1.24) or *1298C* allele (OR = 0.24; 95% CI: 0.06–0.97). However, no modifying effect of the *MTHFR C677T* genotype was noted on the association between folate intake and breast cancer risk. There was some evidence of a joint association of folate and the *A1298C* genotype, but the sample size was not large enough to examine this association.

We did not find an overall reduced risk of breast cancer associated with *MTHFR 677TT* or *1298CC* genotypes, which is not consistent with the British study of breast cancer (17) and some of the previous studies for other cancers. Two of three case-control studies of colorectal cancer and the *MTHFR C677T* polymorphism observed an overall reduction in risk associated with the *TT* genotype (10, 11), as did studies of oral cancer (23) and adult acute lymphocytic leukemia (24). We also found a similar weak association among women with high intake of folate and folate cofactors. However, other studies of colorectal cancer (12), colorectal adenoma (13, 14, 25), gastric cancer (9), lung cancer (26), and acute myeloid leukemia (24) found no association or an increased risk of cancer for individuals with the *TT* genotype. Our observation for a stronger inverse association of folate intake and breast cancer risk

Table 4 Joint association of folate and *MTHFR C677T* genotype with breast cancer risk stratified by cofactor intake among Chinese women, Shanghai Breast Cancer Study, 1996–1998

Folate cofactor intake	Folate intake, odds ratio (95% confidence interval)[a]			*P* for trend	*P* for interaction
	T_3 (high)	T_2	T_1		
Vitamin B_{12}[b]					
Low					
CC	1.00 (reference)	1.71 (0.74–3.94)	1.77 (0.73–4.34)	0.25	
CT	1.62 (0.70–3.73)	1.36 (0.61–3.05)	1.74 (0.75–4.06)	0.79	
TT	0.83 (0.28–2.45)	0.86 (0.31–2.40)	2.36 (0.90–6.16)	0.11	0.56
Medium					
CC	1.00 (reference)	1.88 (0.97–3.67)	2.11 (0.97–4.59)	0.11	
CT	1.01 (0.56–1.81)	1.69 (0.92–3.11)	1.81 (0.89–3.68)	0.20	
TT	0.84 (0.38–1.87)	1.77 (0.83–3.83)	3.00 (1.18–7.61)	0.03	0.45
High					
CC	1.00 (reference)	0.96 (0.51–1.81)	1.10 (0.58–1.73)	0.39	
CT	1.00 (0.58–1.73)	1.22 (0.67–2.22)	1.26 (0.65–2.48)	0.88	
TT	0.61 (0.30–1.23)	2.33 (1.03–5.26)	1.25 (0.46–3.37)	0.12	0.02
Vitamin B_6[c]					
Low					
CC	1.00 (reference)	0.77 (0.16–3.86)	1.24 (0.26–5.91)	0.30	
CT	0.70 (0.09–5.37)	1.11 (0.23–5.41)	1.43 (0.31–6.76)	0.26	
TT	2.19 (0.09–52.31)	0.74 (0.13–4.27)	1.89 (0.38–9.39)	0.06	0.70
Medium					
CC	1.00 (reference)	1.45 (0.68–3.07)	1.67 (0.71–3.94)	0.24	
CT	0.84 (0.37–1.91)	1.31 (0.63–2.74)	1.18 (0.54–2.59)	0.57	
TT	0.66 (0.22–1.97)	1.37 (0.60–3.17)	1.74 (0.65–4.71)	0.02	0.70
High					
CC	1.00 (reference)	1.61 (0.88–2.96)	1.28 (0.29–5.59)	0.04	
CT	1.27 (0.84–1.92)	1.26 (0.75–2.11)	1.01 (0.33–3.04)	0.44	
TT	0.71 (0.41–1.22)	2.50 (1.11–5.63)	0.68 (0.12–3.73)	0.23	0.13
Methionine[d]					
Low					
CC	1.00 (reference)	2.17 (0.50–9.38)	1.87 (0.43–8.18)	0.88	
CT	2.82 (0.60–13.29)	2.00 (0.47–8.51)	2.06 (0.48–8.88)	0.98	
TT	3.24 (0.48–22.00)	1.56 (0.33–7.45)	2.89 (0.64–13.04)	0.41	0.98
Medium					
CC	1.00 (reference)	1.21 (0.61–2.40)	1.99 (0.91–4.37)	0.09	
CT	0.99 (0.48–2.05)	1.47 (0.76–2.84)	1.75 (0.85–3.64)	0.30	
TT	0.96 (0.37–2.48)	1.70 (0.77–3.73)	2.05 (0.74–5.67)	0.09	0.91
High					
CC	1.00 (reference)	1.71 (0.90–3.23)	1.57 (0.51–4.83)	0.16	
CT	1.08 (0.69–1.66)	1.31 (0.76–2.24)	1.16 (0.52–2.59)	0.82	
TT	0.59 (0.33–1.05)	1.84 (0.83–4.08)	1.53 (0.50–4.66)	0.03	0.19

[a] All odds ratios are adjusted for age, waist-to-hip ratio, age at first live birth, physical activity, menopausal status, and total energy, meat, and intake of the other two cofactors.
[b] *P* for three-way interaction, 0.47.
[c] *P* for three-way interaction, 0.24.
[d] *P* for three-way interaction.

among women with the TT genotype is supported by the majority of studies examining a similar association for other cancers (10–12, 14, 15, 23), and is consistent with the role of folate in breast carcinogenesis. We, and others, have previously found a decreased risk of breast cancer among those with high intake level of folate (19, 27–33). Low folate intake is associated with an increased misincorporation of uracil and chromosome breaks (34, 35) and aberrant DNA methylation (35, 36). The critical factor in breast carcinogenesis may be an appropriate balance between the availability of for DNA methylation and 5,10-methylene-*S*-adenosylmethioninetetrahydrofolate for DNA synthesis. It is plausible that individuals with the 677TT genotype are particularly susceptible to the carcinogenic consequences of folate insufficiency. This genotype, in the presence of low folate, is associated with higher levels of homocysteine, lower levels of methylated folates and, therefore, reductions in genomic DNA methylation (37, 38). Our finding for a positive association of *C677T* genotype with low folate intake (OR = 2.51, 95% CI: 1.37–4.60) appears to support this notion. Conversely, in folate-replete conditions, the availability of 5,10-methylenetetrahydrofolate for nucleotide synthesis may be adequate or increased for these individuals because of the genetically determined decreased activity of MTHFR. This could explain the lower risk of this genotype among those with high folate levels in this (OR = 0.70, 95% CI: 0.40–1.23 for *677TT*) and other studies (10, 12, 14, 15). Therefore, the effect of *MTHFR* on breast cancer risk in a particular population may depend on the intake level of folate in that population. With increased folic acid fortification in the United States population, the general intake of folate may be higher than that from the Chinese, whose folate intake is primarily obtained from unfortified diets. This may explain, in part, the overall absence

of association of *MTHFR* genotype with breast cancer risk in our study.

The relationship between folate metabolism and carcinogenesis is likely to be a complex biological sum of genetic and nutritional differences. In our study, the association of folate and breast cancer risk was similar for all genotypes when intakes of vitamin B_{12}, B_6, or methionine were low. Vitamin B_{12}, vitamin B_6, and methionine all have important roles in one-carbon metabolism; vitamin B_{12} is a cofactor for the transfer of the methyl group from folate to methionine, vitamin B_6 is a coenzyme for the formation of 5,10-methylene-5,10-methylenetetrahydrofolate and the catabolism of homocysteine, and methionine is the precursor for *S*-adenosylmethionine. It is possible that below a certain intake threshold of vitamin B_{12} and methionine, the effect of *MTHFR C677T* genotype or folate intake is reduced or negated and that once this threshold is surpassed, both folate and *MTHFR* genotype have a greater impact on breast cancer risk. The inverse association with breast cancer risk among those with a high vitamin B_6 intake was unexpected and cannot be readily explained by the above rationale. This finding needs to be re-evaluated in future studies.

As with any case-control study, the potential for selection and recall biases must be considered. However, selection bias is unlikely to be a major issue in this study; both cases (91%) and controls (90%) had very high participation rates. Not only did this study have a high participation rate, it also had a high blood collection rate (>80%). Although it is possible that cases and controls may have had differentially recalled intakes of foods that contributed to the nutrients in this study, fruit and vegetable intake, the major contributors to folate, methionine, and vitamin B_6 intakes, did not significantly differ between cases and controls. Approximately 50% of cases were interviewed within 15 days of diagnosis and the majority (80%) were interviewed within 4 months, thus reducing potential recall bias attributable to dietary change related to a diagnosis of cancer. In addition, recall of diet would unlikely be related to *MTHFR* genotype and, therefore, could not account for the associations we observed in this study. Additionally, misclassification in the assessment of folate intake may have occurred from using the Unites States Department of Agriculture food composition database. However, any such misclassification would be nondifferential between cases and controls and would usually result in a bias toward the null. Confounding by other factors is always a concern in epidemiological studies. We observed little confounding when we carefully adjusted for known risk factors. Although it is possible that residual confounding may still exist, for example, from dietary factors not considered in this study, additional factors are not likely to explain the strength of the observed associations. Other strengths of our study include the population-based design, the estimation of folate intake in a population of nonusers of alcohol and vitamin supplements, and the large sample size that facilitated examination of modifying effects.

In summary, we found that, although there was no overall relationship between *MTHFR* genotype and breast cancer risk, women with low intake of folate and who are homozygous for the *MTHFR 677T* polymorphism may be at substantially increased risk for breast cancer. Our data also suggest this association may be further modified by vitamin B_{12}, vitamin B_6, and methionine intake. This study adds support to the literature that one-carbon metabolism and *MTHFR* polymorphisms have a role in carcinogenesis and may be important in breast carcinogenesis.

References

1. Kim, Y. I. Folate and carcinogenesis: evidence, mechanisms, and implications. J. Nutr. Biochem., *10:* 66–88, 1999.

2. Mason, J. B., and Choi, S. W. Folate and carcinogenesis: developing a unifying hypothesis. Adv. Enzyme Regul., *40:* 127–141, 2000.

3. Matthews, R. G., Sheppard, C., and Goulding, C. Methylenetetrahydrofolate reductase and methionine synthase: biochemistry and molecular biology. Eur. J. Pediatr., *157* (Suppl. 2): S54–S59, 1998.

4. Fodinger, M., Horl, W. H., and Sunder-Plassmann, G. Molecular biology of 5,10-methylenetetrahydrofolate reductase. J. Nephrol., *13:* 20–33, 2000.

5. Bailey, L. B., and Gregory, J. F., III. Folate metabolism and requirements. J. Nutr., *129:* 779–782, 1999.

6. Frosst, P., Blom, H. J., Milos, P., Goyette, P., Sheppard, C. A., Matthews, R. G., Boers, G. J. H., den Heijer, M., Kluijtmans, L. A. J., van der Heuvel, L. P., and Rozen, R. A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. Nat. Genet., *10:* 111–113, 1995.

7. Weisberg, I., Tran, P., Christensen, B., Sibani, S., and Rozen, R. A second genetic polymorphism in methylenetetrahydrofolate reductase (MTHFR) associated with decreased enzyme activity. Mol. Genet. Metab., *64:* 169–172, 1998.

8. Weisberg, I. S., Jacques, P. F., Selhub, J., Bostom, A. G., Chen, Z. T., Ellison, R. C., Eckfeldt, J. H., and Rozen, R. The 129XA -> C polymorphism in methylenetetrahydrofolate reductase (MTHFR): *in vitro* expression and association with homocysteine. Atherosclerosis, *156:* 409–415, 2001.

9. Shen, H., Xu, Y., Zheng, Y., Qian, Y., Yu, R., Qin, Y., Wang, X., Spitz, M. R., and Wei, Q. Polymorphisms of 5,10-methylenetetrahydrofolate reductase and risk of gastric cancer in a Chinese population: a case-control study. Int. J. Cancer, *95:* 332–336, 2001.

10. Chen, J., Giovannucci, E., Kelsey, K., Rimm, E. B., Stampfer, M. J., Colditz, G. A., Spiegelman, D., Willett, W. C., and Hunter, D. J. A methylenetetrahydrofolate reductase polymorphism and the risk of colorectal cancer. Cancer Res., *56:* 4862–4864, 1996.

11. Ma, J., Stampfer, M. J., Giovannucci, E., Artigas, C., Hunter, D. J., Fuchs, C., Willett, W. C., Selhub, J., Hennekens, C. H., and Rozen, R. Methylenetetrahydrofolate reductase polymorphism, dietary interactions, and risk of colorectal cancer. Cancer Res., *57:* 1098–1102, 1997.

12. Slattery, M. L., Potter, J. D., Samowitz, W., Schaffer, D., and Leppert, M. Methylenetetrahydrofolate reductase, diet, and risk of colon cancer. Cancer Epidemiol. Biomarkers Prev., *8:* 513–518, 1999.

13. Chen, J., Giovannucci, E., Hankinson, S. E., Ma, J., Willett, W. C., Spiegelman, D., Kelsey, K. T., and Hunter, D. J. A prospective study of methylenetetrahydrofolate reductase and methionine synthase gene polymorphisms, and risk of colorectal adenoma. Carcinogenesis (Lond.), *19:* 2129–2132, 1998.

14. Ulrich, C. M., Kampman, E., Bigler, J., Schwartz, S. M., Chen, C., Bostick, R., Fosdick, Beresford, A. A., Yasui, Y., and Potter, J. D. Colorectal adenomas and the C677T MTHFR polymorphism: evidence for gene-environment interaction? Cancer Epidemiol. Biomarkers Prev., *8:* 659–668, 1999.

15. Levine, A. J., Siegmund, K. D., Ervin, C. M., Diep, A., Lee, E. R., Frankl, H. D., and Haile, R. W. The methylenetrahydrofolate reductase 677C->T polymorphism and distal colorectal adenoma risk. Cancer Epidemiol. Biomarkers Prev., *9:* 657–663, 2000.

16. Gershoni-Baruch, R., Dagan, E., Israeli, D., Kasinetz, L., Kadouri, E., and Friedman, E. Association of the C677T polymorphism in the *MTHFR* gene with breast and/or ovarian cancer risk in Jewish women. Eur. J. Cancer, *36:* 2313–2316, 2000.

17. McGlynn, K. A., Wang, L., Patrick-Acevedo, N. Y., Strachan, S. D., and Kruger, W. D. Methylenetetrahydrofolate reductase, methionine synthase, folate, alcohol and breast cancer. The 91st Annual AACR Meeting, April 1–5, 2000, San Francisco, CA, p. 92.

18. Sharp, L., Little, J., Schofield, A. C., Pavlidou, E., Cotton, S. C., Miedzybrodzka, Z., Baird, J. O., Haites, N. E., Heys, S. D., and Grubb, D. A. Folate and breast cancer: the role of polymorphisms in methylenetetrahydrofolate reductase (MTHFR). Cancer Lett., *181:* 65–71, 2002.

19. Shrubsole, M. J., Jin, F., Dai, Q., Shu, X. O., Potter, J. D., Hebert, J. R., Gao, Y. T., and Zheng, W. Dietary folate intake and breast cancer risk: results from the Shanghai Breast Cancer Study. Cancer Res., *61:* 7136–7141, 2001.

20. Gao, Y. T., Shu, X. O., Dai, Q., Potter, J. D., Brinton, L. A., Wen, W., Sellers, T. A., Kushi, L. H., Ruan, Z., Bostick, R. M., Jin, F., and Zheng, W. Association of menstrual and reproductive factors with breast cancer risk: results from the Shanghai Breast Cancer Study. Int. J. Cancer, *87:* 295–300, 2000.

21. Shu, X. O., Yang, G., Jin, F., Liu, D., Kushi, L., Wen, W., Gao, Y. T., and Zheng, W. Validity and reproducibility of the food frequency questionnaire used in the Shanghai Women's Health Study. Eur. J. Clin. Nutr., *58:* 17–23, 2004.

22. Willett, W. C. Nutritional Epidemiology, Vol. 30, Ed. 2. New York, United States of America: Oxford University Press, pp. 291–292, 1998.

23. Weinstein, S. J., Gridley, G., Harty, L. C., Diehl, S. R., Brown, L. M., Winn, D. M., Bravo-Otero, E., and Hayes, R. B. Folate intake, serum homocysteine and methylenetetrahydrofolate reductase (MTHFR) C677T genotype are not associated with oral cancer risk in Puerto Rico. J. Nutr., *132:* 762–767, 2002.

24. Burbee, D. G., Forgacs, E., Zochbauer-Muller, S., Shivakumar, L., Fong, K., Gao, B., Randle, D., Kondo, M., Virmani, A., Bader, S., Sekido, Y., Latif, F., Milchgrub, S., Toyooka, S., Gazdar, A. F., Lerman, M. I., Zabarovsky, E., White, M., and Minna, J. D. Epigenetic inactivation of RASSF1A in lung and breast cancers and malignant phenotype suppression. J. Natl. Cancer Inst., *93:* 691–699, 2001.

25. Nomura, M., Watari, J., Yokota, K., Saitoh, Y., Obara, T., and Kohgo, Y. Morphogenesis of nonpolypoid colorectal adenomas and early carcinomas assessed by cell proliferation and apoptosis. Virchows Arch., *437:* 17–24, 2000.

26. Shen, H., Spitz, M. R., Wang, L. E., Hong, W. K., and Wei, Q. Polymorphisms of methylene-tetrahydrofolate reductase and risk of lung cancer: a case-control study. Cancer Epidemiol. Biomarkers Prev., *10:* 397–401, 2001.

27. Graham, S., Hellmann, R., Marshall, J., Freudenheim, J., Vena, J., Swanson, M., Zielezny, M., Nemoto, T., Stubbe, N., and Raimondo, T. Nutritional epidemiology of postmenopausal breast cancer in western New York. Am. J. Epidemiol., *134:* 552–566, 1991.

28. Freudenheim, J. L., Marshall, J. R., Vena, J. E., Laughlin, R., Brasure, J. R., Swanson, M. K., Nemoto, T., and Graham, S. Premenopausal breast cancer risk and intake of vegetables, fruits, and related nutrients. J. Natl. Cancer Inst., *88:* 340–348, 1996.

29. Thorand, B., Kohlmeier, L., Simonsen, N., Croghan, C., and Thamm, M. Intake of fruits, vegetables, folic acid and related nutrients and risk of breast cancer in postmenopausal women. Public Health Nutr., *1:* 147–156, 1998.

30. Ronco, A., De Stefani, E., Boffetta, P., Deneo-Pellegrini, H., Mendilaharsu, M., and Leborgne, F. Vegetables, fruits, and related nutrients and risk of breast cancer: a case-control study in Uruguay. Nutr. Cancer, *35:* 111–119, 1999.

31. Zhang, S., Hunter, D. J., Hankinson, S. E., Giovannucci, E. L., Rosner, B. A., Colditz, G. A., Speizer, F. E., and Willett, W. C. A prospective study of folate intake and the risk of breast cancer. J. Am. Med. Assoc., *281:* 1632–1637, 1999.

32. Rohan, T. E., Jain, M. G., Howe, G. R., and Miller, A. B. Dietary folate consumption and breast cancer risk. J. Natl. Cancer Inst., *92:* 266–269, 2000.

33. Levi, F., Pasche, C., Lucchini, F., and La Vecchia, C. Dietary intake of selected micronutrients and breast-cancer risk. Int. J. Cancer, *91:* 260–263, 2001.

34. Blount, B. C., Mack, M. M., Wehr, C. M., MacGregor, J. T., Hiatt, R. A., Wang, G., Wickramasinghe, S. N., Everson, R. B., and Ames, B. N. Folate deficiency causes uracil misincorporation into human DNA and chromosome breakage: implications for cancer and neuronal damage. Proc. Natl. Acad. Sci. USA, *94:* 3290–3295, 1997.

35. Kim, Y. I., Pogribny, I. P., Basnakian, A. G., Miller, J. W., Selhub, J., and Mason, J. B. Folate deficiency in rats induces DNA strand breaks and hypomethylation within the *p53* tumor suppressor gene. Am J. Clin. Nutr., *65:* 46–52, 1997.

36. Rampersaud, G. C., Kauwell, G. P., Hutson, A. D., Cerda, J. J., and Bailey, L. B. Genomic DNA methylation decreases in response to moderate folate depletion in elderly women. Am. J. Clin. Nutr., *72:* 998–1003, 2000.

37. Jacques, P. F., Bostom, A. G., Williams, R. R., Ellison, R. C., Eckfeldt, J. H., Rosenberg, I. H., Selhub, J., and Rozen, R. Relation between folate status, a common mutation in methylenetetrahydrofolate reductase, and plasma homocysteine concentrations. Circulation, *93:* 7–9, 1996.

38. Friso, S., Choi, S. W., Girelli, D., Mason, J. B., Dolnikowski, G. G., Bagley, P. J., Olivieri, O., Jacques, P. F., Rosenberg, I. H., Corrocher, R., and Selhub, J. A common mutation in the 5,10-methylenetetrahydrofolate reductase gene affects genomic DNA methylation through an interaction with folate status. Proc. Natl. Acad. Sci. USA, *99:* 5606–5611, 2002.

Appendix 2

Shrubsole MJ, Shu XO, Ruan ZX, Cai Q, Cai H, Niu Q, Gao YT, Zheng W.
MTHFR genotypes and breast cancer survival after surgery and chemotherapy: A report from the Shanghai Breast Cancer Study. 2004. *Clinical Cancer Research* (submitted).

MTHFR genotypes and breast cancer survival after surgery and chemotherapy: A report from the Shanghai Breast Cancer Study

Martha J. Shrubsole[1,2,3,4,5], Xiao Ou Shu[1,2,3,4,5], Zhi Xian Ruan[6], Qiuyin Cai[1,2,3,4,5], Hui Cai[1,2,3,4], Qi Niu[1,2,3], Yu-Tang Gao[6], Wei Zheng[1,2,3,4,5]

[1]Department of Medicine, Division of Internal Medicine, [2]Vanderbilt Center for Health Services Research, [3]Vanderbilt-Ingram Comprehensive Cancer Center, [4]Vanderbilt School of Medicine, Nashville, Tennessee; [5]VA TN Valley Geriatric Research, Education, and Clinical Center (GRECC), Nashville, TN.; and [6]Department of Epidemiology, Shanghai Cancer Institute, Shanghai, China.

Keywords: breast cancer; survival; MTHFR; chemotherapy

Running title: MTHFR and breast cancer survival

Grant Support: This research was supported by USPHS Grant RO1CA64277 from the National Cancer Institute. Dr. Shrubsole is supported in part by DAMD17-02-1-0606.

Requests for reprints: Martha J. Shrubsole, Center for Health Services Research, Division of Internal Medicine, Department of Medicine, School of Medicine, Vanderbilt University Medical Center, 1161 21st Avenue South, S-1121 Medical Center North, Nashville, TN 37232-2587. Email: martha.shrubsole@vanderbilt.edu

ABSTRACT

Purpose: Methylenetetrahydrofolate reductase (MTHFR) regulates the pool of intracellular folates for DNA synthesis and methylation. Sequence variations in the *MTHFR* gene (nucleotides 677 (C→T) and 1298 (A→C)) result in allozymes with decreased activity. The *677 TT* genotype is associated with increased toxicity of methotrexate and increased clinical response to 5-fluorouracil in treatment of cancers including breast cancer.

Experimental Design: We evaluated *MTHFR* genotypes and breast cancer survival in a cohort of 1067 Chinese women who were diagnosed with breast cancer between 1996 and 1998 and received surgery and chemotherapy. Life table method was used to calculate 5 year survival rates and Cox proportional hazards models were used to estimate hazard ratios (HR) and 95% confidence intervals (CI) after adjusting for potential confounding factors.

Results: Median follow-up time was 5.2 years. Five-year survival was 84.6% for this study population. 66% of the patients carried a *677 T* allele and 31% carried a *1298 C* allele. We found that overall five-year survival after diagnosis of breast cancer did not differ significantly across all genotypes (85.3% for *677 CC* and 83.8% for *677 TT*; 83.8% for *1298 AA* and 79.1% for *1298 CC*). However, carrying the *677 T* allele was associated with increased risk of death for subjects with late stage disease (stages III-IV) (HR=1.85, 95% CI: 0.82-4.19 for *TT* vs. *CC*, p for trend=0.13), particularly among those who had survived the first year (HR=2.50, 95% CI: 1.07-5.85, p for trend=0.04). The *A1298C* genotypes were not significantly associated with risk of death.

Conclusion: This study suggests that the *MTHFR C677T* polymorphisms may affect long-term survival from advanced breast cancer.

INTRODUCTION

Methylenetetrahydrofolate is an important enzyme in folate metabolism. It irreversibly converts 5,10-methylene tetrahydrofolate (THF) to 5-methyl THF which provides the methyl group for the de novo synthesis of methionine synthase and DNA methylation (1). It also helps determine the folate levels available for DNA synthesis and repair (2). Two common polymorphisms in the *MTHFR* gene have been identified (3;4). The 677C →T polymorphism codes for an alanine to valine substitution in the N-terminal catalytic domain and results in an allozyme with approximately 65% and 30% of the activity of the wild-type protein for heterozygotes and homozygotes, respectively (3). The A→C polymorphism at nucleotide 1298 codes for an alanine to glutamine substitution in the C-terminal regulatory domain (4). Individuals homozygous for the 1298C allele have approximately the same enzyme activity as those heterozygous for the 677T allele (4;5).

Adjuvant chemotherapy greatly improves relapse-free and overall survival of breast cancer patients (6-8). A classical treatment regime for metastatic disease is a combination of cyclophosphamide, methotrexate (MTX), and fluorouracil (5FU). Both MTX and 5FU exert their anti-neoplastic activities through folate-pathway inhibition. It has been reported that the *MTHFR 677TT* genotype is associated with increased toxicity of MTX and increased clinical response to 5FU in treatment of leukemia, and cancers of the breast, ovary, and colorectum (9-12). The relationship between *MTHFR* genotypes and survival from breast cancer has not been previously investigated. In this study, we examine this association among a cohort of Chinese breast cancer patients who had participated in the Shanghai Breast Cancer Study and had received adjuvant chemotherapy as part of their treatment for breast cancer.

PATIENTS AND METHODS

Participants and Data Collection: The Shanghai Breast Cancer Study is a population-based case-control study that recruited breast cancer patients during August 1996 through March 1998. Eligible cases were residents of urban Shanghai aged 25-64 years with a newly diagnosed breast cancer who had no prior history of cancer. Cases were identified through a rapid case-ascertainment system supplemented by the population-based Shanghai Tumor Registry. A total of 1602 cases were found to be eligible for the study, and, of these, 1459 (91.1%) completed in person interviews, 17 (1.1%) were deceased before interview, 109 (6.8%) refused to participate and 17 (1.1%) could not be located. Information on cancer diagnosis, disease stage (TNM), and treatment were abstracted from medical records. Chemotherapy regimen information was not collected in the study. Blood samples were available for 1193 women (81.8%). These samples were processed within 6 hours of collection and stored at -70° C. For nearly 50% of the cases, blood sample collection and in-person interviews were completed before any cancer therapy.

During March 2000 to January 2003, the 1459 participating cases were followed up to collect data on disease progression, recurrence, survival status, and quality of life among survivors. In-person (n=1241, 85.0%) or telephone interviews (n=49, 3.4%) were completed by the patient or by next of kin for deceased patients (n=197). The survival status of the remaining 169 cases was established by linkage to the vital statistics registry at the Shanghai Center for Disease Control and Prevention (SCDCP). Four cases did not have sufficient information for linkage and were excluded from the study. Through the linkage, 43 deaths were identified and information on dates and causes of death were obtained. To allow a delay in the transmittal of death information to the SCDCP, we assigned the December 30, 2002 as the date of last contact to the cases (n=122) whose file did not match those in the vital statistics registry. This study was

approved by the Institutional Review Board of Vanderbilt University and all of the participating institutions; consent was obtained for all study participants.

Laboratory methods: Genomic DNA was extracted from blood samples with the Puregene® DNA isolation Kit (Gentra Systems, Minneapolis, MN) following the manufacturer's protocol. Genotyping for the *MTHFR C677T* and *A1298C* polymorphisms were performed using PCR-RFLP methods described elsewhere (13). Briefly, the primers for *C677T* analysis were: 5'-TGAAGGAGAAGGTGTCTGCGGGA-3' (exonic) and 5'-AGGACGGTGCGGTGAGAGTG-3' (intronic). The primers for *A1298C* analysis were: 5'-GGGAGGAGCTGACCAGTGCAG-3' and 5'-GGGGTCAGGCCAGGGGCAG-3'. The PCR reactions were performed in a Biometra® TGradient Thermocycler. Each 20 ml of PCR mixture contained 10 ng DNA, 1x PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 9.0), 1.5 mM $MgCl_2$, 0.2 mM each of dNTP, 0.5 mM of each primer, and 1 unit of Taq DNA polymerase. The reaction mixture was initially denatured at 94°C for 3 min. For *C677T* polymorphisms, PCR was performed in 30 cycles of 94°C for 45 sec, 65°C for 45 sec, and 72°C for 45 sec. For *A1298C* polymorphisms, PCR was performed in 35 cycles of 94°C for 45 sec, 65°C for 45 sec, and 72°C for 45sec. The PCR was completed by a final extension cycle at 72°C for 7 min.

For *C677T* polymorphisms, each PCR product (10 µl) was digested with 10 units of *Hinf* I at 37°C for 3 hours. The DNA fragments were then separated using 3% agarose gel and detected by ethidium bromide staining. The C→T substitution at nucleotide 667 creates a *Hinf* I digestion site. The PCR product (198 bp) with *T* allele was digested to 2 fragments (175 bp and 23 bp), whereas the PCR product with wild type C allele cannot be cut by *Hinf* I. For *A1298C* polymorphisms, each PCR product (10 µl) was digested with 5 units of *Fnu4H* I at 37°C for 3 hours followed by 3% agarose gel electrophoresis and ethidium bromide staining. The A→C

substitution at nucleotide 1298 creates a *Fnu4H* I site. The PCR product (138 bp) with *C* allele was digested to 2 fragments (119 bp and 19 bp), whereas the PCR product with wild type *A* allele cannot be cut by *Fnu4H* I.

Quality control (QC) samples were included in various batches of samples assayed for the polymorphisms. The consistency rate was 98.5% in 119 QC samples that were repeated in the genotyping assays with their identities unknown to lab staff. Excluding a few participants for whom sufficient DNA was not available or for whom the genotyping assay failed, genotyping data were obtained from 1038 and 1045 participants for *C677T* and *A1298C* polymorphisms, respectively.

Data Analysis: All analyses were restricted to the 1366 (93.6%) who were known to have received both surgery and chemotherapy as part of their treatment for breast cancer. *MTHFR* analyses were further restricted to the women who had genotyping data. Overall survival time was calculated as the time from diagnosis to death from any cause and censored at date of last contact. Kaplan-Meier survival method was used to calculate the 1,3, and 5-year survival rates and the Log Rank Test was applied to test the differences in survival across different genotypes. Cox proportional hazards model was used to estimate hazard ratios (HR) and their 95% confidence intervals (CI) after adjusting for use of radiotherapy and Tamoxifen and known prognostic factors including TNM stage and age. Age was included as a continuous variable throughout, and categorical variables were treated as indicator variables in the model. Stratified analyses were used to evaluate the potential modifying effect of age, ER/PR status, and TNM stage on risk associated with *MTHFR* genotypes. All statistical tests were based on two-sided probabilities using SAS, version 8.2 (SAS Institute, Inc., Cary, NC).

RESULTS

Characteristics of participants and overall 5-year survival rates by characteristic are presented in Table 1 for the entire cohort and for the subset with *MTHFR* data. Distributions and 5-year survival rates were consistent between both groups. Of those with known ER and PR status, most were positive for ER or PR. Overall 5-year survival was 84.63%. Survival was somewhat lower for those who were older at diagnosis. The majority of patients received radiotherapy (66.2%) or took Tamoxifen (70.1%) (data not shown in table).

In Table 2, the frequencies of *MTHFR* alleles and genotypes are presented among all the study participants and by TNM stage and ER/PR status. The frequencies of the *677T* and *1298C* alleles were 0.41 and 0.18 respectively. Neither the *C677T* nor the *A1298C* genotypes were significantly associated with the stage at diagnosis or ER/PR status. The distribution of *A1298C* genotypes was similar overall and across TNM stage and ER/PR status. Disease stage and ER/PR status also did not appear to be influenced by the combined genotype of *C677T* and *A1298C*. No significant differences in genotype distribution were observed. The distributions of the *MTHFR* genotypes did not differ from the predicted distribution under Hardy-Weinberg equilibrium (p=0.83 for *C677T* and p=0.07 for *A1298C* polymorphisms).

In Table 3, overall 5-year survival rates and hazard ratios are presented by *MTHFR* genotype. Median follow-up of the patient cohort was 5.2 years (data not shown in table). 5-year overall survival rates were 85.3%, 84.8%, and 83.8% for women with the 677 CC, CT, and TT genotypes and 83.8%, 86.6%, and 79.1% for women with the 1298 AA, AC, and CC genotypes.

Overall risks of death according to stage at diagnosis and *MTHFR* genotype are presented in Table 4. Among those diagnosed with a late stage breast cancer, there was a suggestive increased risk of death with increasing frequency of *677T* alleles (p for trend=0.13). When overall survival was examined among those who had survived at least one year, presence of the

677 TT genotype was associated with a more-than-double risk of death among those with late-stage disease (OR=2.50, 95% CI=1.07-5.85, p for trend= 0.04). This association was stronger for women who had survived at least 2 years (OR=2.99, 95% CI: 3.98 (1.14-7.87), p for trend=0.03). Conversely and non-significantly, risk of death was decreased among those with the *TT* genotype and early-stage disease (OR=0.58, 95% CI=0.29-1.14, p for trend=0.13). There was no evidence of a survival difference by the presence of the *1298C* allele. *MTHFR C667T* results for stage III were similar to the combined analysis of stages III and IV (data not shown in table). Sample sizes did not permit separate analysis of the *A1298C* genotypes or stage IV breast cancer alone.

The Kaplan-Meier survival functions for overall survival by *MTHFR* genotypes and TNM stages are presented in Figure 1. Consistent with the results shown in Table 4, patients with a late stage cancer and who carried the 677TT genotype had the poorest survival. The difference was particularly evident after 3 years of follow-up.

DISCUSSION

In this population-based breast cancer cohort study, we found that *MTHFR* genotypes were not associated with overall survival in the entire cohort. However, the *677 TT* genotype was associated with substantially poorer long-term survival among those with late stage disease. This is the first report suggesting that *MTHFR* genotype affects survival from breast cancer.

MTHFR has a critical role in folate metabolism. Decreased activity of MTHFR increases the folate pool available for DNA synthesis and cell proliferation. The *MTHFR 677T* polymorphism results in an allozyme with lower activity; it is approximately 70% reduced *in vitro* for homozygotes (3). Folate pathway inhibitors such as MTX and 5-FU are important chemotherapeutic drugs used in the treatment of breast cancer. The primary target of MTX is

dihydrofolate reductase and, thus, MTX increases the intracellular pool of dihydrofolate, an inhibitor of MTHFR (14) while simultaneously functioning as an anti-folate and a TS inhibitor (15). 5FU, another common drug for treatment of breast cancer and TS inhibitor, must form a ternary complex with TS and 5,10-methylene THF(16). Situations in which the availability of 5,10-methylene THF is increased, including decreased activity of MTHFR, will result in a more stable complex and maximal inhibition of TS, thus decreasing proliferation and increasing cytotoxicity. This is supported by the report of greater chemosensitivity of cells with 677 TT MTHFR (17) and the greater response to 5FU of metastatic colorectal cancer patients with *677TT* genotype (12). Recently, Sohn et al (17) transfected the breast cancer cell line MDA-MB-435 with *677CC* or *677TT* human MTHFR cDNA to examine chemosensitivity to MTX and 5-FU, as measured by the percentage of cell survival. As expected, cells expressing the 677T MTHFR had 35% lower MTHFR activity, and proportionally less methyl THF, more formyl THF, more methylene THF, and grew more rapidly than the cells expressing the C677 MTHFR. However, the cells with 677T MTHFR were more chemosensitive to 5FU but less chemosensitive to MTX treatment. Although we do not have specific chemotherapy information for each patient in this study, based on data collected from a recent survey in Shanghai, over 84% of breast cancer patients received 5FU and 39% MTX. Therefore, we would expect that in the study population, patients who carry the *677TT* genotype would be more sensitive to chemotherapy, in general, and more likely to have better survival, if the chemotherapy dose is appropriate. This hypothesis has been supported by one study conducted in colon cancer patients who received 5FU (12), although there was no association between survival from colon cancer and the *677T* allele in another smaller study (18). Contrary to this hypothesis, we found that women with late-stage disease and the *TT* genotype had poorer survival, particularly in the later years of follow-up, possibly due to the chronic toxic effects of chemotherapy. In one report of 6 breast cancer patients who received cyclophophamide, MTX, and 5FU and experienced grade IV toxicity, 5 had the *677TT* genotype (19). Increased acute MTX toxicity has also been reported for TT

carriers with ovarian cancer (11), CML and bone marrow transplant (20), acute leukemia (9), and rheumatoid arthritis (21). In a French-Canadian study of 201 children with acute lymphoblastic leukemia, the presence of the *677T* allele was associated with a higher probability of relapse or death (22). Survival was even poorer if the individuals had both the *677T* allele and were homozygous for the triple repeat of the 5'UTR thymidylate synthase polymorphism, which is associated with increased TS levels.

Although we knew that all women in this study received chemotherapy as part of their treatment for breast cancer, specific chemotherapy data were not available. Because the type of chemotherapy prescribed is unlikely to be related to *MTHFR* genotype, confounding effect is not a concern for the study. However, if the survival differences associated with *MTHFR* genotypes are modified by chemotherapy type, the lack of this type of data in our population could have affected our results. In an on-going study of breast cancer survival in Shanghai, a similar sample of 341 women were recruited during 2003. Over 84% of patients received 5FU and 39% received MTX as part of their therapy. Specific regimens were as follows: CMF 32%, 31% CEF, and 1% AC. Because stage of disease (local vs. distant) is one of the most important factors that determines the type of therapy received, we examined the effect of genotype within stage. The results of this study suggest that there might be an interaction between *MTHFR* genotype and chemotherapy, and this hypothesis will need to be tested in future studies with both genotype and treatment information. Selection and survival biases were minimized by the population-based design, high participation rate of the identified cases, and high follow-up rate. Likewise there was also a high participation rate for DNA samples and participation was not likely affected by genotype. We also cannot exclude chance as a reason for our findings.

In summary, we found that the *MTHFR 677 TT* genotype was associated with poor long-term survival for women with late-stage breast cancer, but did not affect survival from early-stage disease. We did not find an association between *MTHFR A1298C* and breast cancer survival. This is the first report of *MTHFR* genotype and breast cancer survival. The importance of folate-pathway inhibiting drugs in adjuvant chemotherapy for breast cancer and the high prevalence of the *MTHFR* genotypes have potential implications for the treatment of breast and other cancers including adjustment of chemotherapy dose. These results are intriguing and warrant further investigation in larger studies with specific data on chemotherapy courses.

ACKNOWLEDGMENTS

We are grateful to the patients and research staff who participated in the Shanghai Breast Cancer Study and to Qing Wang for her excellent technical assistance.

REFERENCES

1. Matthews RG, Sheppard C, Goulding C. Methylenetetrahydrofolate reductase and methionine synthase: biochemistry and molecular biology. Eur J Pediatr 1998; 157 Suppl 2: S54-S59.

2. Bailey LB, Gregory JF, III. Folate metabolism and requirements. J Nutr 1999; 129(4): 779-82.

3. Frosst P, Blom HJ, Milos P et al. A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. Nat Genet 1995; 10: 111-3.

4. Weisberg I, Tran P, Christensen B, Sibani S, Rozen R. A second genetic polymorphism in methylenetetrahydrofolate reductase (MTHFR) associated with decreased enzyme activity. Mol Genet Metab 1998; 64(3): 169-72.

5. Weisberg IS, Jacques PF, Selhub J et al. The 1298A -> C polymorphism in methylenetetrahydrofolate reductase (MTHFR): in vitro expression and association with homocysteine. Atherosclerosis 2001; 156(2): 409-15.

6. Early Breast Cancer Trialists' Collaborative Group. Systemic treatment of early breast cancer by hormonal, cytotoxic, or immune therapy. 133 randomised trials involving 31,000 recurrences and 24,000 deaths among 75,000 women. Lancet 1992; 339(8785): 71-85.

7. Early Breast Cancer Trialists' Collaborative Group. Systemic treatment of early breast cancer by hormonal, cytotoxic, or immune therapy. 133 randomised trials involving 31,000 recurrences and 24,000 deaths among 75,000 women. Lancet 1992; 339(8784): 1-15.

8. Early Breast Cancer Trialists' Collaborative Group. Polychemotherapy for early breast cancer: an overview of the randomised trials. Lancet 1998; 352(9132): 930-42.

9. Chiusolo P, Reddiconto G, Casorelli I et al. Preponderance of methylenetetrahydrofolate reductase C677T homozygosity among leukemia patients intolerant to methotrexate. Ann Oncol 2002; 13(12): 1915-8.

10. Toffoli G, Veronesi A, Boiocchi M, Crivellari D. MTHFR gene polymorphism and severe toxicity during adjuvant treatment of early breast cancer with cyclophosphamide, methotrexate, and fluorouracil (CMF). Ann Oncol 2000; 11(3): 373-4.

11. Toffoli G, Russo A, Innocenti F et al. Effect of methylenetetrahydrofolate reductase 677C-->T polymorphism on toxicity and homocysteine plasma level after chronic methotrexate treatment of ovarian cancer patients. Int J Cancer 2003; 103(3): 294-9.

12. Cohen V, Panet-Raymond V, Sabbaghian N, Morin I, Batist G, Rozen R. Methylenetetrahydrofolate reductase polymorphism in advanced colorectal cancer: a novel genomic predictor of clinical response to fluoropyrimidine-based chemotherapy. Clin Cancer Res 2003; 9(5): 1611-5.

13. Shrubsole MJ, Gao YT, Cai Q et al. MTHFR polymorphisms, dietary folate intake, and breast cancer risk: results from the Shanghai Breast Cancer Study. Cancer Epidemiol Biomarkers Prev 2004; 13(2): 190-6.

14. Matthews RG, Daubner SC. Modulation of methylenetetrahydrofolate reductase activity by S-adenosylmethionine and by dihydrofolate and its polyglutamate analogues. Adv Enzyme Regul 1982; 20: 123-31.

15. Allegra CJ, Chabner BA, Drake JC, Lutz R, Rodbard D, Jolivet J. Enhanced inhibition of thymidylate synthase by methotrexate polyglutamates. J Biol Chem 1985; 260(17): 9720-6.

16. Chu E, Callender MA, Farrell MP, Schmitz JC. Thymidylate synthase inhibitors as anticancer agents: from bench to bedside. Cancer Chemother Pharmacol 2003; 52 Suppl 1: S80-S89.

17. Sohn KJ, Croxford R, Yates Z, Lucock M, Kim YI. Effect of the methylenetetrahydrofolate reductase C677T polymorphism on chemosensitivity of colon and breast cancer cells to 5-fluorouracil and methotrexate. J Natl Cancer Inst 2004; 96(2): 134-44.

18. Wisotzkey JD, Toman J, Bell T, Monk JS, Jones D. MTHFR (C677T) polymorphisms and stage III colon cancer: response to therapy. Mol Diagn 1999; 4(2): 95-9.

19. Lieber CS, Packer L. S-Adenosylmethionine: molecular, biological, and clinical aspects--an introduction. Am J Clin Nutr 2002; 76(5): 1148S-50S.

20. Ulrich CM, Yasui Y, Storb R et al. Pharmacogenetics of methotrexate: toxicity among marrow transplantation patients varies with the methylenetetrahydrofolate reductase C677T polymorphism. Blood 2001; 98(1): 231-4.

21. Urano W, Taniguchi A, Yamanaka H et al. Polymorphisms in the methylenetetrahydrofolate reductase gene were associated with both the efficacy and the toxicity of methotrexate used for the treatment of rheumatoid arthritis, as evidenced by single locus and haplotype analyses. Pharmacogenetics 2002; 12(3): 183-90.

22. Krajinovic M, Lemieux-Blanchard E, Chiasson S, Primeau M, Costea I, Moghrabi A. Role of polymorphisms in MTHFR and MTHFD1 genes in the outcome of childhood acute lymphoblastic leukemia. Pharmacogenomics J 2004; 4(1): 66-72.

Table 1: Overall Survival by Selected Breast Cancer Prognosis Factors Among Breast Cancer Patients

	All subjects[a]				Subjects with *MTHFR* data			
Characteristic at Baseline	Subjects n (%)	No. of death	5-year survival rate (%)	P-value	Subjects n (%)	No. of death	5-year survival rate (%)	P-value
	1366	222	84.2		1067	172	84.6	
Age at Diagnosis								
<42	322 (24)	52	85.2		263 (25)	44	84.9	
42-46	338 (25)	40	88.3		268 (25)	29	89.4	
47-52	345 (25)	61	82.4		267 (25)	48	82.4	
53-64	361 (26)	69	81.4	0.06	269 (25)	51	81.8	0.05
Education								
< Middle school	165 (12)	34	79.8		131 (12)	29	79.2	
Middle school	587 (43)	95	83.9		474 (45)	75	84.5	
>Middle school	614 (45)	92	85.7	0.18	462 (43)	68	86.3	0.14
TNM								
0-I	335 (24)	21	94.0		266 (25)	17	94.2	
II	790 (58)	112	86.3		622 (58)	87	86.6	
III -IV	161 (12)	68	58.3		123 (12)	51	59.4	
Unknown	80 (06)	21	75.8	<0.0001	56 (5)	17	72.8	<0.0001
ER								
Positive	622 (46)	95	85.3		487 (46)	75	85.5	
Negative	360 (26)	59	83.8		279 (26)	41	85.8	
Unknown	384 (28)	68	82.9	0.63	301 (28)	56	82.2	0.42
PR								
Positive	613 (45)	91	85.5		492 (46)	75	85.4	
Negative	354 (26)	61	83.0		266 (25)	39	85.9	
Unknown	399 (29)	70	83.3	0.50	309 (29)	58	82.3	0.38

[a] Includes all women who had both surgery and chemotherapy as part of their initial breast cancer treatment.

Table 2. *MTHFR* allele and genotype frequencies according to breast cancer prognostic factors. Shanghai Breast Cancer Study, 1996-2002.

MTHFR	All subjects	TNM Stage[1]				ER/PR Status[2]		
		0-I	IIa	IIb	III-IV	ER+/PR+ n (%)	ER+/PR- or ER-/PR+ n (%)	ER-/PR- n (%)
Allele Frequency								
677T	0.41	0.40	0.43	0.38	0.41	0.39	0.40	0.44
1298C	0.18	0.18	0.18	0.19	0.16	0.17	0.40	0.44
Genotype[3]								
C677T, n (%)								
CC	355 (34.2)	94 (36.2)	112 (29.5)	93 (41.3)	42 (35.6)	148 (38.0)	47 (34.1)	57 (31.1)
CT	507 (48.8)	126 (48.5)	208 (54.9)	92 (40.9)	56 (47.5)	178 (45.6)	71 (51.4)	92 (50.3)
TT	176 (17.0)	40 (15.4)	59 (15.6)	40 (17.8)	20 (16.9)	64 (16.4)	20 (14.5)	34 (18.6)
p[4]			0.06				0.47	
A1298C, n (%)								
AA	717 (68.6)	180 (68.7)	256 (68.1)	156 (67.2)	87 (71.9)	271 (69.5)	88 (65.7)	125 (67.6)
AC	287 (27.5)	72 (27.5)	106 (28.2)	66 (28.4)	29 (24.0)	102 (26.1)	42 (31.3)	49 (26.5)
CC	41 (3.9)	10 (3.8)	14 (3.7)	10 (4.3)	5 (4.1)	17 (4.4)	4 (3.0)	11 (5.9)
p[4]			0.98				0.59	
AC/CC	328 (31.4)	82 (31.3)	120 (31.9)	76 (32.8)	34 (28.1)	119 (30.4)	46 (34.3)	60 (32.4)
p[4]			0.84				0.70	
A1298C and C677T, n (%)								
AA and CC	185 ()	51 (19.9)	56 (15.2)	48 (21.7)	22 (19.0)	78 (20.6)	22 (16.8)	30 (16.6)
AA and CT	343 ()	85 (33.2)	138 (37.4)	63 (28.5)	42 (36.2)	125 (33.0)	44 (33.6)	59 (32.6)
AA and TT	172 ()	40 (15.6)	57 (15.5)	40 (18.1)	20 (17.2)	63 (16.6)	19 (14.5)	34 (28.8)
AC/CC and CC	157	40 (15.6	55 (14.9)	43 (19.5)	19 (16.4)	66 (17.4)	23 (17.6)	26 (14.4)
AC/CC and CT/TT	143	40 (15.6)	63 (17.1)	27 (12.2)	13 (11.2)	47 (12.4)	23 (17.6)	32 (17.7)
p[4]			0.36				0.63	

1 Stage is unknown in 80 participants.
2 ER/PR status is unknown in 402 participants
3 C677T genotyping was not available for 29 participants; A1298C genotyping was not available for 22 participants
4 P-value is calculated from chi-square test

Table 3. Five year relapse, metastasis and death in association with MTHFR genotypes. Shanghai Breast Cancer Study, 1996-2002.

MTHFR genotype	Total subjects	No. of deaths	Overall survival		
			5-year (95% CI)	HR (95% CI)[1]	HR (95% CI)[2]
	1067	172	84.2		
C677T[3]					
CC	355	57	85.3 (81.5-89.0)	1.00 (Ref)	1.00 (Ref)
CT	507	80	84.8 (81.6-87.9)	1.00 (0.71-1.40)	1.13 (0.80-1.60)
TT	176	29	83.8 (78.3-89.3)	1.04 (0.67-1.63)	0.93 (0.59-1.46)
			P=0.96		
A1298C[3]					
AA	717	118	83.8 (81.1-86.6)	1.00 (Ref)	1.00 (Ref)
AC	287	43	86.6 (82.6-90.5)	0.88 (0.62-1.25)	0.89 (0.62-1.26)
CC	41	9	79.1 (66.1-92.1)	1.33 (0.68-2.63)	1.08 (0.55-2.15)
			P=0.59		
AC/CC	328	52	85.6 (81.8-89.5)	0.94 (0.67-1.30)	0.92 (0.66-1.27)
			P=0.73		
A1298C and C677T					
AA and /CC	185	28	84.6 (79.4-89.9)	1.00 (Ref)	1.00 (Ref)
AA/CT	343	59	83.4 (79.4-87.4)	1.15 (0.73-1.80)	1.12 (0.71-1.75)
AA/TT	172	28	84.0 (78.5-89.6)	1.08 (0.64-1.82)	0.88 (0.52-1.49)
AC/CC and CC	163	28	85.4 (79.9-90.9)	1.10 (0.65-1.85)	0.90 (0.53-1.52)
AC/CC and CT/TT	153	21	86.8 (81.4-92.2)	0.89 (0.51-1.57)	0.98 (0.56-1.74)
			P=0.86		

1 Adjusted for age at diagnosis.
2 Adjusted for TNM stage, age, and radiotherapy and Tamoxifen.

Table 4. Risk of death among breast cancer patients associated with MTHFR polymorphisms and TNM stage and according to duration of follow-up. Shanghai Breast Cancer Study, 1996-2002.

MTHFR genotype	TNM Stage 0-II		TNM Stage III-IV	
	No. of deaths/ all subjects	HR (95% CI)	No. of deaths/ all subjects	HR (95% CI)
Overall risk of death				
C677T[3]				
CC	38/299	1.00 (Ref)	15/42	1.00 (Ref)
CT	49/426	0.88 (0.58-1.35)	21/56	1.44 (0.72-2.89)
TT	15/139	0.73 (0.40-1.33)	11/20	1.85 (0.82-4.19)
p for trend		0.30		0.13
A1298C[3]				
AA	75/592	1.00 (Ref)	36/87	1.00 (Ref)
AC/CC	32/278	0.91 (0.60-1.38)	14/34	0.67 (0.35-1.27)
		0.64		0.22
Risk of death within 1 year				
C677T[3]				
CC	3/299	1.00 (Ref)	2/42	1.00 (Ref)
CT/TT	8/565	1.38 (0.36-5.20)	5/76	1.28 (0.25-6.70)
p for trend		0.64		0.77
A1298C[3]				
AA	8/592	1.00 (Ref)	6/87	1.00 (Ref)
AC/CC	3/278	0.79 (0.21-2.98)	1/34	0.31 (0.04-2.67)
		0.73		0.29
Risk of death after 1 year				
C677T[3]				
CC	35/296	1.00 (Ref)	13/40	1.00 (Ref)
CT	45/422	0.88 (0.56-1.36)	16/51	1.35 (0.62-2.92)
TT	11/135	0.58 (0.29-1.14)	11/20	2.50 (1.07-5.85)
p for trend		0.13		0.04
A1298C[3]				
AA	64/584	1.00 (Ref)	29/81	1.00 (Ref)
AC/CC	29/275	0.92 (0.59-1.43)	13/33	0.80 (0.40-1.59)
		0.72		0.52

1 HR are adjusted for age and radiation and Tamoxifen therapies

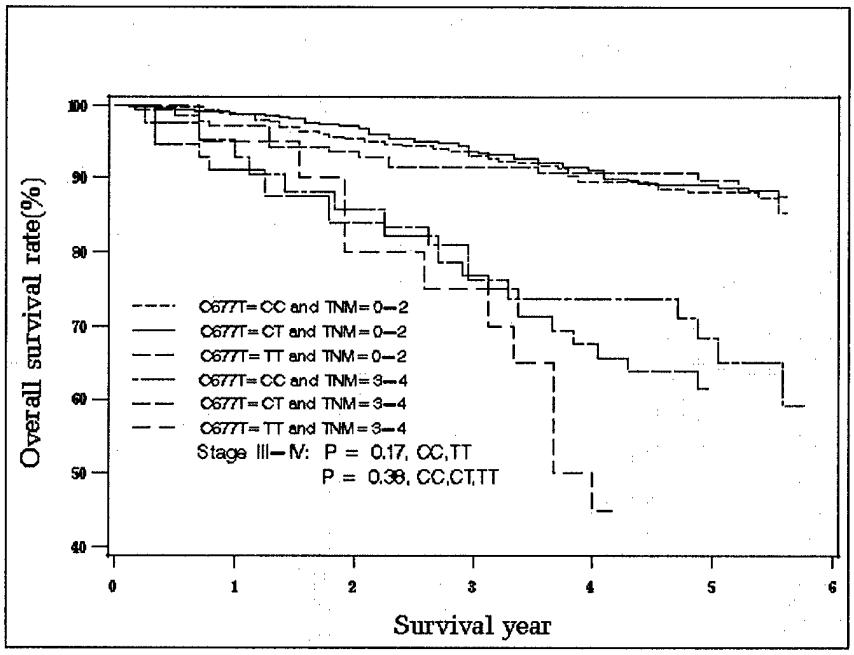

Figure 1. Kaplan-Meier estimates of overall survival from breast cancer by *MTHFR C677T* genotype and TNM stage. Shanghai Breast Cancer Study, 1996-2002. Ps shown are from the log-rank test.